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Antibody-dependent cell-mediated cytotoxicity antibody responses to inactivated and live-attenuated influenza vaccination in children during 2014-15

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Abstract

Background—Seasonal influenza vaccines aim to induce strain-specific neutralizing antibodies. Non-neutralizing antibodies may be more broadly cross-reactive and still protect through mechanisms including antibody-dependent cell-mediated cytotoxicity (ADCC). Influenza vaccines may stimulate ADCC antibodies in adults, but whether they do so in children is unknown. Here we examined how vaccination affects cross-reactive ADCC antibody responses in children after receipt of inactivated trivalent vaccine (IIV3) or quadrivalent live-attenuated vaccine (LAIV4).

Methods—Children aged 5–17 were recruited in fall 2014 to provide pre- and post-vaccination serum samples. Children aged 5–9 received LAIV4 based on then-current recommendation, and older children were randomly assigned to IIV3 or LAIV4. We used microtiter-plate-based flow cytometry with an NK cell line to examine ADCC antibody responses to the 2014–15 H3N2 vaccine component (A/Texas/50/2012 [TX12]) and a drifted strain, A/Switzerland/9715293/2013 (SW13). Responses were stratified by two-season (2013–14 and 2014–15) vaccine sequence.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Results—Eighty-five children received LAIV4 and 45 received IIV3. Prevaccination ADCC activity was highest in children who had received any vaccine in the prior season. Increase in ADCC antibody responses against the vaccine strain TX12 following vaccination was greatest for participants who received IIV3 in 2014–15 and LAIV4 in the prior season (geometric mean fold rise [MFR]=1.6, 95% CI 1.23 – 2.11). This group also had a detectable ADCC response to the drifted SW13 strain. There was a modest ADCC response against SW13 in LAIV4 recipients who were unvaccinated in the previous season (MFR=1.18, 95% CI 1.10 – 1.25). There were no significant changes in 2014–15 ADCC response to vaccination among children who had received IIV3 in 2013–14.

Conclusions—Vaccinating children with IIV3 after prior receipt of LAIV4 generated a modest increase in ADCC antibodies, including some cross-reactivity with an emerging drift variant. Other vaccine-induced ADCC responses were minimal and not affected by vaccine type or sequence.

Keywords

ADCC; influenza vaccine; antigenic drift; children

1. Introduction

Influenza vaccines are optimized to elicit neutralizing antibodies, which primarily recognize the variable globular head domain of the hemagglutinin (HA) attachment protein. Vaccine strains are selected primarily on their ability to elicit strong hemagglutination-inhibiting (HI) antibodies in serum against viruses that are projected to be dominant in circulation in the coming season [1]. Early challenge studies suggested that an HI titer of 1:40 is associated with 50% reduction in influenza incidence in healthy adults (sometimes referred to as protection of 50% of subjects), but more recent studies have shown that this threshold is most likely not a valid correlate of protection [2–4]. Because most neutralizing antibodies induced by infection and/or current vaccines are highly strain-specific, antigenic match between the vaccine and circulating strains also affects vaccine-induced protection. Vaccine-induced antibodies may have reduced activity against antigenically drifted viruses, or may be directed against epitopes that are not present or accessible on circulating viruses, contributing to reduced protection.

Antibodies can mediate important immune effector functions beyond neutralization [5] [6] [7]. For example, interactions with Fc receptors (FcR) may contribute to the ability of broadly neutralizing antibodies directed against the conserved HA stalk to provide protection against influenza virus infection *in vivo* [8; 9]. Once bound to antigen, antibody Fc regions can mediate complement fixation [10], phagocytosis [11; 12], and/or antibody-dependent cell- mediated cytotoxicity (ADCC) [13]; the latter 2 functions require interaction with FcR on immune cells.

Recent studies have shown that seasonal influenza vaccines stimulate antibodies capable of mediating ADCC (“ADCC antibodies”) [14–16]. ADCC antibodies need not be neutralizing and may cross-react against a range of virus strains and subtypes. It has therefore been proposed that vaccine-induced ADCC antibodies could provide a measure of protection

against antigenically divergent viruses, including pandemic viruses, when most neutralizing antibodies, directed as they are against the variable globular head of HA, would fail to do so [7; 17].

In 2014, an H3N2 antigenic drift variant belonging to clade 3c2.A, represented by A/Hong Kong/4801/2014, emerged and became dominant during the 2014–15 Northern Hemisphere influenza season. The H3N2 vaccine component for that season, A/Texas/50/2012 (TX12), was a poor match for these circulating drift variants, and vaccine effectiveness was low [18]. Northern Hemisphere vaccines for the 2015–16 influenza season were updated to include A/Switzerland/9715293/2013 (SW13), a clade 3c3.A virus more closely matched to the drift variants that emerged in the previous season. Here we took advantage of this naturally emerging influenza antigenic variation and vaccine update to examine the capacity of influenza vaccines to elicit cross-reactive ADCC antibody responses in children. We reasoned that, while previous studies had shown that influenza vaccination can boost ADCC antibodies in adults, such responses could rely on pre-existing immunity and therefore that vaccination might not effectively stimulate ADCC antibodies in children, whose influenza exposure histories are more limited. We analyzed ADCC antibody responses to 2014–15 season vaccination in children receiving either live attenuated or inactivated vaccines. Children's responses were also stratified by prior season (2013–14) vaccination (live attenuated, inactivated, or none) to assess repeat vaccination effects.

2. Materials and Methods

2.1. Study population and serum collection

Parents of children 5–17 years old were recruited in the fall of 2014 by mail and telephone to participate in a serological vaccine study [19] of antibody responses after receipt of quadrivalent live attenuated inactivated influenza vaccine (LAIV4) or trivalent inactivated influenza vaccine (IIV3) Participants were children (n=130) who (a) participated in a similar serologic influenza vaccine study in the previous 2013– 14 influenza season [20], or (b) lived in Marshfield, Wisconsin or surrounding area where annual studies of influenza vaccine effectiveness were conducted [18]. Recruitment was restricted to this population because they were living in the surveillance area for laboratory-confirmed influenza infections during the prior influenza season, and vaccination information for this population is captured through a validated vaccination registry [21].

Participants aged 5–8 years received LAIV4 according to then-current recommendations from the U.S. Advisory Committee on Immunization Practices (ACIP), while 9–17-year-olds were randomized to receive either LAIV4 or IIV3 [22]. Participants had a baseline serum blood draw at the time of vaccination and a post-vaccination serum blood draw at 28 days. HI antibody responses to 2014–15 influenza A vaccine viruses in these participants, including the H3N2 strain TX12, have been described previously [23].

2.2. Informed consent

Study procedures were approved by the Marshfield Clinic Institutional Review Board. Informed consent was obtained from the parents/guardians of all participants and assent was obtained from children aged ≥7 years.

2.3. Antibody-dependent cell-mediated cytotoxicity (ADCC) assay

We examined serum antibodies' ability to trigger degranulation by natural killer (NK) cells in the presence of HA proteins as a proxy for ADCC. Degranulation is detected by the presence of CD107a on the surface of NK cells. The frequency of NK cells expressing CD107a in the presence of HA protein and participant serum (subtracting the frequency of NK cells that spontaneously express CD107a in the absence of antigen) is proportional to the magnitude of ADCC antibody responses [24]. We measured HA- and serum-specific degranulation by NK reporter cells using a modification of a protocol described previously [24]. Briefly, 96-well plates were coated overnight with trimeric recombinant HA TX12 or SW13; proteins were expressed in a baculovirus expression system (kindly provided by Centers for Disease Control and Prevention, USA) [25]. Wells were then washed multiple times with phosphate-buffered saline (PBS) to remove unbound proteins. A 1:10 dilution of heat- inactivated serum (56°C for 1 hour) was then added to each well and incubated at 37°C for 2 hours. For endpoint titers a serum dilution series was prepared (1:10,1:50,1:100,1:500,1:1000). Wells were again washed repeatedly with PBS. Next, 2×10^5 NK cells expressing CD16 (cell line KHYG-1, kindly provided by Dr. David Evans of the University of Wisconsin-Madison) were added to each well in RPMI medium containing 10% fetal calf serum (Hyclone, Logan, UT), together with anti-human-CD107a-APC-H7 (H4A3 clone, BD Biosciences, San Jose, CA), 5 μg/ml Brefeldin A (Sigma, St. Louis, MO) and 5 μg/ml Monensin (Golgi Stop, BD Biosciences, San Jose, CA) [26]. Plates were incubated for 5 hours at 37°C, after which time cells were incubated with the following antibodies for 30 min at room temperature: anti-CD16 (clone 3G8, BD Biosciences, San Jose, CA) antiNKG2A PC7 (clone Z199, Beckman Coulter, Brea, CA) and Live Dead Stain Near IR (ThermoFisher Scientific, Grand Island, NY). Finally, cells were fixed with 1% paraformaldehyde and acquired on an LSRII flow cytometer (BD Biosciences). Wells without serum added were used as negative controls in each assay, as influenza-naïve human serum was not available. A combination of phorbol 12-myristate 13-acetate (PMA) and Ionomycin was used as a positive control for the assay. KHYG-1 cells are used here to provide a consistent population of effector cells and control for variability in activity of primary NK cells among participants. Data were analyzed using FlowJo Version 10.0. The endpoint antibody titer was defined as the serum dilution at which the frequency of NK cell degranulation was equal to the no-antigen control.

2.4. Statistical analyses

Participants were first grouped for analysis by type of vaccine received in the 2013–14 influenza season: IIV3 (n = 58), LAIV4 (n = 51), or none (n = 21; Table 1). Serologic endpoints were defined as (1) ADCC antibody activity measured against the vaccine strain TX12 and the drift variant SW13 at baseline and (2) mean fold rise in ADCC antibody activity against these proteins from baseline to post-vaccination in 2014–15.

Comparisons were tested using Student's T test, ANOVA, linear regression or Chi-Squared as indicated. All tests involving multiple comparisons were adjusted using Bonferroni's method of adjusting for multiple comparisons. Statistical analyses and plots were conducted or produced in R version 3.3.0 using ggplot2 (https://www.R-project.org; [27] and Graphpad Prism version 6 (GraphPad Software, La Jolla, California). Because this was an exploratory study, sample size calculations were not performed.

3. Results

3.1. Participant characteristics

The analysis included 130 children, including 85 who received LAIV4 and 45 who received IIV3; 109 (84%) had been vaccinated in the prior season (Table 1). The mean age was 10.8 years and 10 participants (8%) had PCR-confirmed influenza virus infection during the prior season; 9 of these infections were with H1N1pdm09 and one was unsubtypeable [20]. By design, there were significant differences in the number of subjects and mean age in each group.

3.2. Antibodies capable of antibody-dependent cell-mediated cytotoxicity (ADCC) are present at baseline

We measured ADCC antibody responses using a microtiter plate-based assay described previously [24; 28]. This assay quantifies the extent to which participant serum (the source of influenza-specific antibodies) can activate an NK reporter cell line in the presence of plate-bound influenza HA proteins. ADCC activity was detectable in most participants against TX12, SW13, or both viruses prior to vaccination (Figure 1). Baseline ADCC activity was significantly higher among children with prior season vaccination than without: compared to unvaccinated children in 2013–14, mean activity was higher for children who received IIV3 in 2013–14 [34.6% of NK cells responding (95% CI 31.6 – 37.6) $P < 0.001$], and children who received LAIV4 [30.6% (95% CI 26.7 – 34.5) vs. 19.3% (95% CI 13.8 – 24.8) $P = 0.001$]. ADCC activity at baseline did not differ significantly by age or by 2013–14 H1N1pdm09 influenza infection status.

3.3. Vaccine type moderately affects ADCC antibody response

We next determined whether the ADCC response after vaccination was affected by prior season vaccine status and/or modality. Participants who received LAIV4 during in 2013–14 followed by IIV3 in 2014–15 had modest, but statistically significant, increases in ADCC antibodies against the vaccine strain TX12 (mean fold rise in GMT of 1.6; $P=0.045$), but not against the drift variant SW13 (Figure 2). Participants who were unvaccinated during the 2013–14 influenza season and received LAIV4 the following season showed increases in ADCC antibodies against TX12 that were even more modest, but still significant (mean fold rise in GMT of 1.1; $P=0.005$); however there was no measurable increase in ADCC antibodies against SW13 in these participants (Figure 2). There was no significant increase in ADCC antibody frequency after vaccination in any of the other exposure groups.

3.4. ADCC antibody response to vaccination is associated with HI antibody response

HI antibody titers against TX12 at baseline and after IIV or LAIV vaccination in the 2014–15 season were previously reported for participants in this study [23]; unfortunately it was not possible to test SW13. In Figure 3 we replotted these data according to the exposure groups defined above. As observed for ADCC antibody responses, most participants had detectable HI antibodies against TX12 at baseline, which were modestly increased following vaccination in subjects receiving IIV3 (P=0.002 for both IIV3-IIV3 and LAIV4-IIV3). A regression analysis suggested that increases in ADCC antibody levels after vaccination were significantly associated with fold rise in HI titer to the H3N2 vaccine antigen (for ADCC antibodies TX12 P=0.006 and against SW13 P<0.001) and with age under 11 years (significant for ADCC antibodies against SW13 P=0.004 only; TX12 P=1). However, age and rise in HI titer do not fully predict ADCC antibody response (TX12 adj. R^2=0.061; SW13 adj. R^2=0.185).

3.5. Endpoint titration of ADCC antibodies in select participants

To compare the differences in ADCC antibody activity against TX12 and SW13 with greater resolution we determined the endpoint titer of ADCC antibodies in a subset of participants for whom sufficient sample volumes were available. We focused on participants in the LAIV4-IIV3 and LAIV4-LAIV4 groups because receipt of LAIV4 in 2013–14 was associated with the greatest observed increase in ADCC antibody activity in our initial experiments (Figure 2). Of the selected participants who received LAIV4 in 2013–14 and IIV3 in 2014–15, the geometric mean ADCC endpoint titer against TX12 was 14 before vaccination and 67 after vaccination (P=0.04); endpoint titers against SW13 increased from 11 before vaccination to 19 after vaccination, but this change was not statistically significant (P > 0.05; Figure 4). Participants who received LAIV4 in both seasons had no significant changes in ADCC antibody titers against either virus (Figure 4). Notably, participants who received LAIV4 in 2013–14 had higher ADCC antibody titers at baseline than participants who received IIV3 in 2013–14, which might have affected our ability to detect a significant increase in ADCC antibody titers in the LAIV4-LAIV4 group in this subset analysis. Together these results confirmed that in these participants, receipt of LAIV4 followed by IIV3 appeared to increase ADCC antibody responses against the vaccine strain, but not against a contemporaneous drift variant, while our ability to draw conclusions about the effects of LAIV4 vaccination in sequential seasons on ADCC antibody titer were limited.

4. Discussion

ADCC antibodies cannot prevent infection of cells, but can eliminate infected cells, speeding clearance of infection and potentially reducing morbidity and mortality. ADCC has therefore been proposed as a correlate of protective immunity, particularly for newly emerging antigenic variants, against which they could provide a measure of cross-protection in the absence of strain-specific neutralizing antibodies [17]. However, the ability of current vaccine modalities to induce cross-reactive ADCC antibodies is not well characterized. The emergence of SW13 allowed us to examine ADCC antibody responses against a virus antigenically matched to the vaccines received by participants (TX12) and a relevant natural drift variant. Before vaccination with the 2014–15 TX12 vaccine strain, most participants in

the study had baseline ADCC responses against both TX12 and SW13 strains. We observed significant increases in ADCC antibody frequencies after vaccination only in participants who received LAIV4 followed by IIV3, or those who were unvaccinated in 2013–14 and received LAIV4 the following season. In each case, we only observed these statistically significant increases against the TX12 antigen; no group had significant increases in ADCC antibodies against the SW13 drift variant. The ADCC antibody response was correlated with a modest rise in HI titer against the vaccine antigen TX12.

Although we observed statistically significant increases in ADCC antibody activity against TX12, they are quite modest, with mean rises in GMT less than 2-fold. Together these findings suggest that current seasonal influenza vaccines, both live attenuated and inactivated, provide limited boosts to ADCC antibodies in school-aged children.

ADCC antibody responses to primary H1N1pdm09 infection in 5 children were reported by de Vries et al, indicating that children can make robust ADCC antibody responses to homologous virus following natural infection [16]. Most previous studies of ADCC antibody responses to influenza vaccination have focused on adults, who have extensive histories of exposure to influenza [14–16; 29; 30]. Currently licensed seasonal influenza vaccines have been shown to boost cross-reactive ADCC antibodies in adult humans, including in older adults [28; 29]. One recent study used a commercially available reporter assay to examine ADCC responses to H1N1pdm viruses in 20 children and 20 adults given LAIV [31]. They found modest, but detectable, ADCC responses to LAIV vaccination in children (averaging a 2-fold rise relative to baseline), which is consistent with the findings from IIV- and LAIV-vaccinated children we report here. Adults vaccinated with LAIV in this study showed no detectable rise in ADCC antibody activity relative to baseline, and neutralizing antibody titers were not significantly boosted in either adults or children [31] As noted above, we observed an association between response to vaccine antigens (LAIV or IIV) and post-vaccination ADCC antibody activity after vaccination in children, suggesting that in our subjects there was at least a sizeable subset of antibodies capable of inhibiting hemagglutination as well as mediating ADCC. Of note, Zhong et al. observed similar correlations between HI and ADCC antibody responses to influenza vaccination in a cohort of 20 adults given the 2014–15 vaccine (which contained TX12), although twice as many subjects seroconverted for ADCC responses as did for HI responses against the SW13 drift variant (90 vs. 45%; [15]).

The basis for the relatively modest boost in ADCC antibody responses after influenza vaccination in children is not clear. Evidence from animal models may suggest a potential mechanism. Although ADCC antibodies appear to limit the duration of influenza infection in nonhuman primates [24; 28; 32], seasonal vaccines were unable to elicit cross-reactive ADCC antibodies in naïve monkeys [33]. This result suggests that inactivated vaccines may be ineffective at generating antibodies with ADCC activity *de novo*, while vaccination can effectively boost pre-existing ADCC responses. We speculate that the relatively limited influenza exposure histories of children could result in a large proportion of their antibody repertoires targeting the immunodominant globular head of HA and limit their ability to mount ADCC responses to vaccination. In this context we note that, in our study, there was no difference in ADCC antibody response to vaccination in younger (aged 5–9) and older

(aged 9–17) children, as one might expect if accumulating influenza exposures might promote such responses, although responses in our cohort were modest overall and our exploratory study may have been underpowered to observe significant differences by age. This scenario could, however, help explain the close correlation between HI and ADCC responses in these participants and the observation that LAIV followed by IIV3 appeared to modestly boost ADCC antibodies against only the vaccine strain TX12 but not the drift variant SW13. A recent study reported that antibodies targeting the HA globular head (which would be detected in HI assays) were less effective in mediating FcR-dependent effector functions than antibodies that recognized epitopes in the stem [34]. HI antibodies boosted by vaccination in our participants could therefore also mediate the modest and strain-specific ADCC response we observed here, though notably our assays cannot identify epitope specificity of ADCC antibodies. In sum, we do not intend to argue here that ADCC antibodies are strain-specific and not cross-reactive as a general rule, but rather that in humans ADCC antibody specificities, like those of neutralizing antibodies, may be shaped by influenza exposure history and therefore vary with age, repeated vaccination/infection status, and other factors.

Our study has additional important limitations. The plate-based ADCC antibody assay measures CD107a expression on the surface of NK cells after FcγR engagement as a proxy for degranulation and killing of target cells. This method does not examine killing of virus-infected target cells. While we use the trimeric form of HA protein to better represent its native conformation on the cell surface, it is still likely that immobilization of proteins in tissue culture plates exposes epitopes that are not accessible to antibodies *in vivo*. Interpretation of this data is also difficult due to the age distribution of participants with different vaccine exposure histories. Consistent with then-current ACIP recommendations [22], all children aged 5–8 received LAIV4 during the study, causing the LAIV4 groups to have a younger age distribution. As a result, in younger children we were only able to assess responses to the live attenuated vaccine. Despite these limitations, it is nonetheless clear from our study that seasonal influenza vaccines did not effectively boost ADCC antibodies in our school-aged participants.

5. Conclusions

ADCC and other antibody effector functions do not depend on strain-specific virus neutralization, and may therefore play an important role in preventing or ameliorating influenza disease when novel antigenic variants emerge. We examined ADCC responses to influenza vaccination in children for the first time, finding that current influenza vaccines provide only limited boosting to ADCC antibody responses. As noted in the recent National Institute of Allergy and Infectious Disease strategic plan for developing a universal influenza vaccine [35], future studies should prioritize evaluating the role of ADCC and other effector functions mediated via interactions between antibody Fc domains and cellular Fc receptors in cross-reactive immunity to influenza in humans and relevant animal models [6]. Given the existing evidence supporting ADCC in cross-protection against influenza, examining the mechanisms by which vaccines can effectively stimulate antibodies that mediate ADCC as well as other Fc receptor effector functions should also be a priority. Recent findings from small animal models suggesting that broadly neutralizing antibodies against influenza must

also preserve interactions with Fc receptors in order to mediate protection *in vivo* further underscore the importance of these topics [8; 9]. Improved understanding of cross-reactive immunity mediated via Fc-Fc receptor interactions may bring us closer to a broadly cross-reactive influenza vaccine.

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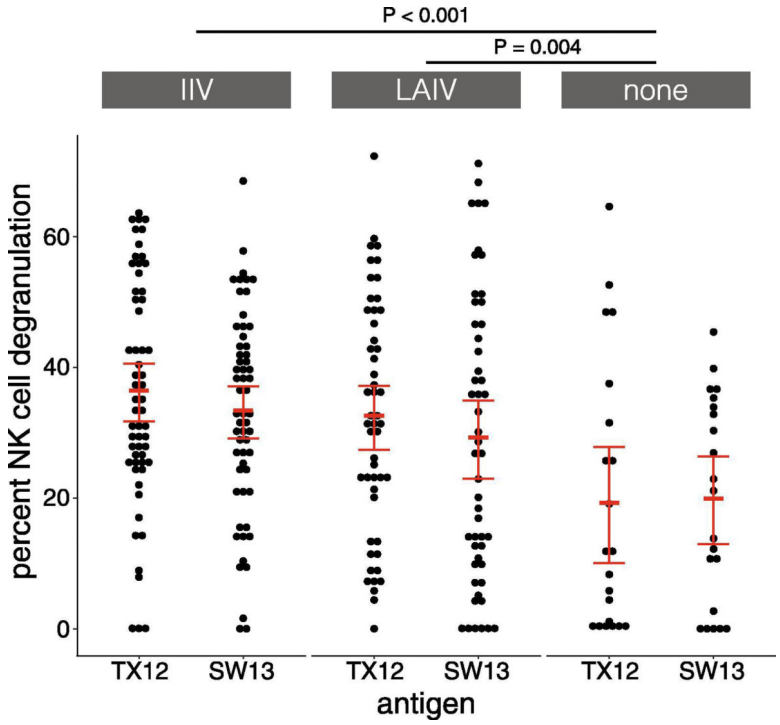

Figure 1. Baseline ADCC antibody responses against A/Texas/50/2012 and A/Switzerland/9715293/2013 in 2014–15 stratified by modality received in the prior season (2013–14).

ADCC antibody responses are reported as the percentage of NK cell degranulation (i.e., CD107a expression) in the presence of serum and antigen for serum samples collected at the time of receipt of 2014–15 influenza vaccine. Red dots and error bars indicate the mean and 95% confidence interval respectively.

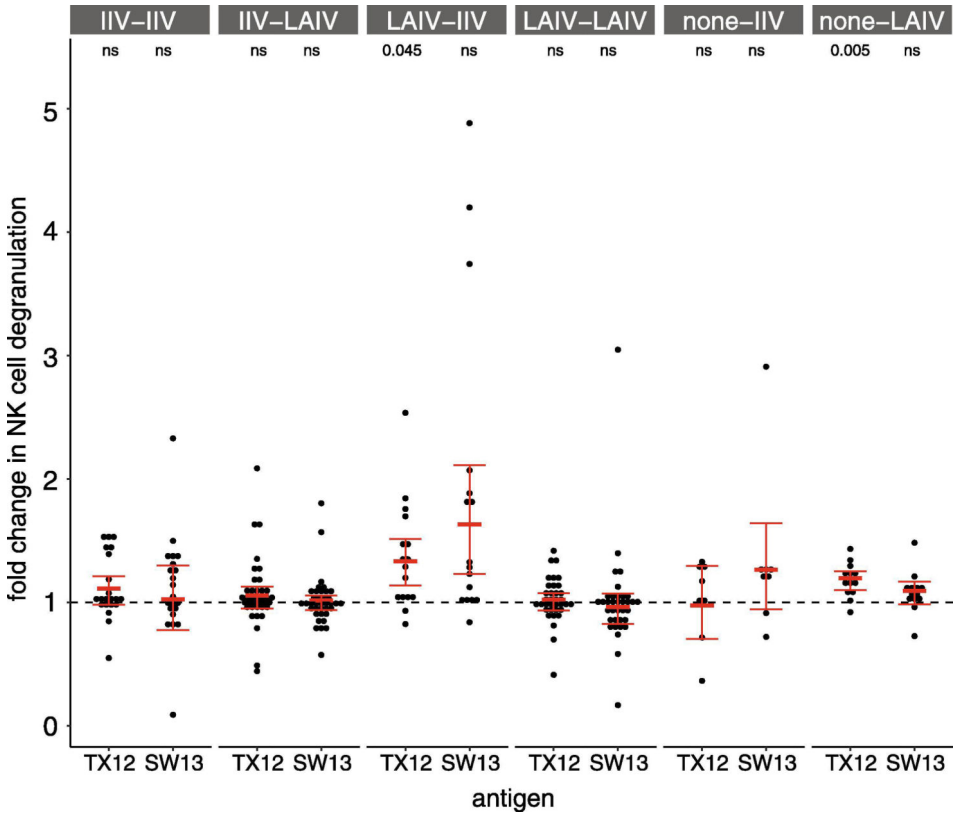

Figure 2. Fold change in ADCC antibodies as measured by NK cell degranulation against A/Texas/50/2012 and A/Switzerland/9715293/2013 stratified by the vaccine modality received in the 2013–14 and 2014–15 influenza seasons.

The fold change in NK cell degranulation is reported as the change from baseline to day 28 post-vaccination. Bars indicate the geometric mean and 95% confidence interval respectively.

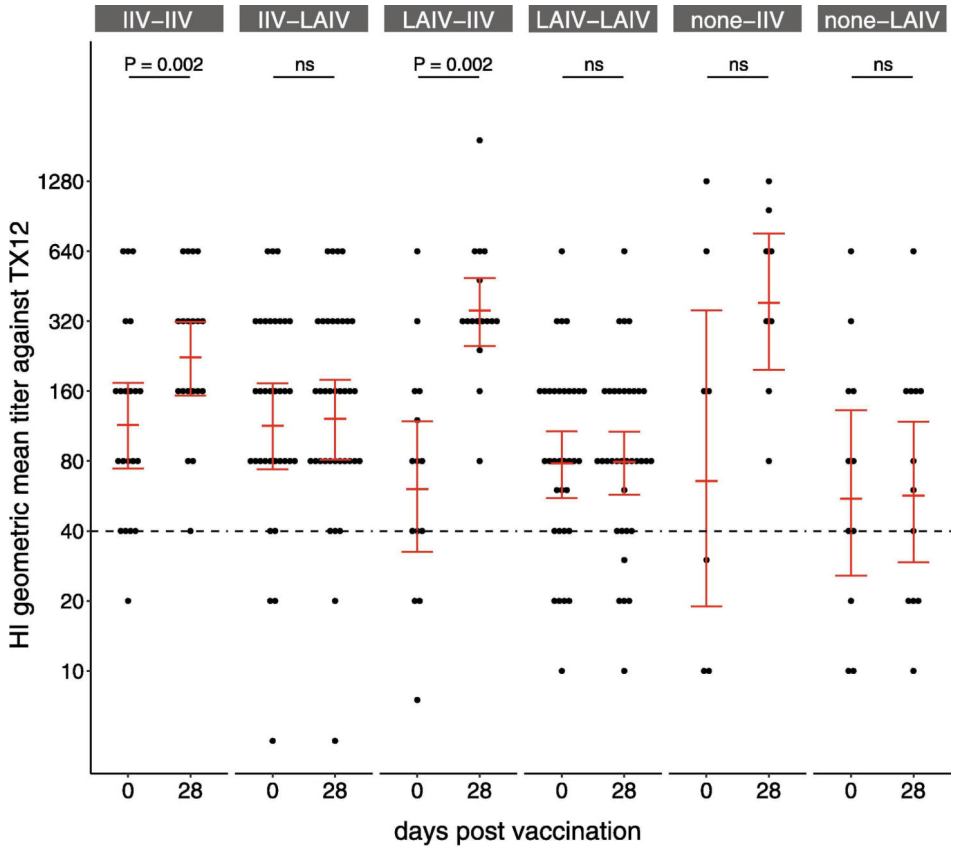

Figure 3. HI antibody responses to A/Texas/50/2012 in study participants.

Changes in HI geometric mean titer were only significant in subjects who received IIV3 followed by IIV3 and LAIV4 followed by IIV3 in the 2013–14 and 2014–15 seasons respectively. Bars indicate the geometric mean and 95% confidence interval respectively.

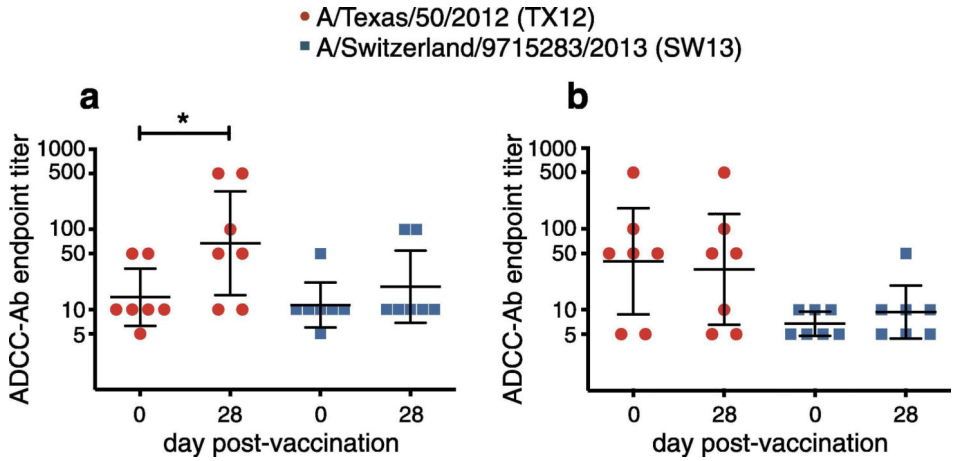

Figure 4. ADCC antibody titers in a subset of subjects vaccinated in consecutive seasons with either LAIV and then IIV or LAIV and then LAIV.

Boosts to ADCC antibody titer were only significant in subjects who received LAIV4 followed by IIV3. 7 subjects receiving LAIV4 then IIV3 (**a**) or LAIV4 in both seasons (**b**) were subsampled from the main cohort for this analysis. Bars indicate the geometric mean and 95% confidence interval.

Table 1:

Study population characteristics

2014-15 vaccine	**IIV3**	**LAIV4**	**overall**
Totals	45	85	130
Age 5-8	0	37	37
Age 9-17	45	48	93
2013-14 vaccine			
IIV3	21	37	58
LAIV4	16	35	51
none	8	13	21
Confirmed influenza infection in 2013-14 *	1	9	10

* 9 of 10 RT-PCR-confirmed influenza infections were subtyped as H1N1pdm09 and one was not subtyped.

We cannot be certain that all potential influenza infections in the study population were captured.